Article

Bacillus velezensis as Antagonist towards *Penicillium roqueforti s.l.* in Silage: *in vitro* and *in vivo* Evaluation

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Abstract: In Belgium, silages are often infected by *Penicillium roqueforti sensu lato* (*s.l.*). These toxigenic fungi are well adapted to silage conditions, and their prevention during feed-out is difficult. *Bacillus velezensis* strain NRRL B-23189 has been reported to inhibit *P. roqueforti s.s.* conidiospore germination *in vitro* by the production of lipopeptides. In the present study, the antagonistic effect of this *B. velezensis* strain towards *P. roqueforti s.l.* was evaluated *in vitro* and *in vivo*. *In vitro*, corn silage conditions were simulated, and the impact of *B. velezensis* culture supernatant or cell suspension on *P. roqueforti s.l.* growth, conidiospore germination and survival and roquefortine C production was evaluated. The antagonism was promising, but growth of *B. velezensis* in corn silage infusion was poor. An *in vivo* experiment with microsilos containing a mixture of perennial ryegrass and white clover artificially contaminated with *P. roqueforti s.l.* was carried out to determine if *B. velezensis* cell suspension could be used as an antagonistic silage inoculant. The *B. velezensis* cell suspension applied was unsuccessful in reducing *P. roqueforti s.l.* numbers at desiling after 56 days compared to no additive application. However, feed-out of the silage was not simulated, so it remains elusive whether or not *B. velezensis* exerts antagonistic activity during this phase.

Keywords: antagonism; *Bacillus velezensis*; *Penicillium roqueforti*; silage; roquefortine C

1. Introduction

In Belgium, silages are often infected by the toxigenic fungal species *Penicillium roqueforti sensu stricto* (*s.s.*) and *P. paneum*. These two fungal species, referred to as *P. roqueforti sensu lato* (*s.l.*), are very closely related and well adapted to silage conditions. Mainly during the feed-out period of silages, when air inevitably regains entrance into the silo, the prevention of their growth and possible mycotoxin production is difficult [1-8].

In the field, growth and mycotoxin production by toxigenic fungi can be prevented by good field management and crop husbandry [9-13], and additionally by the introduction of non-toxigenic isolates of particular fungal species [14-16]. To obtain high quality silage, application of good silo management is crucial [17-21]. As a tool to guide the fermentation process into the desired direction, several types of silage additives are commercially available: fermentation inhibitors (e.g. propionic acid), fermentation stimulants (e.g. homofermentative lactic acid bacteria), aerobic deterioration inhibitors (e.g. heterofermentative lactic acid bacteria), *etc.* Silage additives inhibiting aerobic deterioration may inhibit growth of both yeast and fungi during the feed-out period. Additionally, antagonistic LAB, yeasts and *Bacilli* have been described [18,22-27].

Besides the production of organic acids, LAB can inhibit mycotoxin production by fungi through microbial competition, depletion of nutrients, low pH and production of heat-stable low molecular

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weight metabolites. The genus *Lactobacillus* is well known for its antifungal activity. Lavermicocca, *et al.* [28] found that a ten-fold concentrated culture filtrate of *L. plantarum* 21B possesses efficient antifungal activity against *P. roqueforti s.s.*, *Aspergillus niger*, *A. flavus* and *Fusarium graminearum*, while Ström, *et al.* [29] described *in vitro* broad-spectrum antifungal activity of *L. plantarum* MiLAB 393 against *F. sporotrichioides* and *A. fumigatus*, but not against *P. roqueforti s.s.*.

Yeasts are well-described antagonists of spoilage fungi: Petersson and Schnürer [25],[30,31] have identified *Pichia anomala*, *P. guilliermondii* and *Saccharomyces cerevisiae* yeasts for the biocontrol of fungal growth in ensiled high-moisture cereals. Besides competition for nutrients, the antifungal activity of yeasts can be ascribed to the production of cell wall degrading [30,31].

The production by *Bacillus* species of compounds that display antifungal activity is well documented [32-34]. In plant production, *Bacillus* species including *B. velezensis* have been proven to be promising biocontrol organisms towards fungi infecting growing plants. They can produce cyclic lipopeptides, exerting antifungal effects both in alkaline and acidic conditions. The main families of lipopeptides comprise iturins, surfactins and fengycins, all three exhibiting surfactant properties and antifungal activity [27,35-37].

Chitarra, *et al.* [38] demonstrated that an antifungal compound in the culture supernatant of *Bacillus velezensis* strain NRRL B-23189, able to produce all three families of lipopeptides, inhibits the germination of *P. roqueforti s.s.* conidiospores *in vitro*. Inhibition of conidiospore germination is a beneficial characteristic of an antagonistic organism, since germination is the starting event of a fungus' asexual life cycle [39]. The present study describes both an *in vitro* and *in vivo* experiment aiming to evaluate the antagonistic effect of *B. velezensis* strain NRRL B-23189 (Bv) towards *P. roqueforti s.s.* MUCL 46746 (PR) and *P. paneum* CBS112295 (PP). *In vitro*, corn silage conditions were simulated in corn silage infusion (CSI), while for the *in vivo* experiment a mixture of perennial ryegrass and white clover was artificially contaminated with *P. roqueforti s.s.* or *P. paneum* and treated with Bv cell suspension prior to ensiling in microsilos.

The *in vitro* experiment revealed promising antagonism of Bv towards *P. roqueforti s.l.*, but *in vivo* the bacterial cell suspension could not successfully reduce *P. roqueforti s.l.* numbers in silage. However, it must be noted that the microsilo experiment simulated the silage fermentation process, but not the feed-out of the silage. As an aerobic micro-organism, *B. velezensis* may be able to grow and produce lipopeptides during the feed-out period, but this hypothesis remains to be assessed.

2. Results

2.1. In vitro experiment

2.1.1. Microtiter plate assay for growth monitoring

The results of the microtiter plate assay to monitor *P. roqueforti s.l.* growth are given in Table 1.

Table 1. *In vitro* experiment evaluating the antagonistic effect of *B. velezensis* NRRL B-23189 (Bv) towards *P. roqueforti s.s. MUCL 46746* (PR) and *P. paneum* CBS 112295 (PP): fungal growth registered as optical density at 620 nm (OD_{620}) after five days of incubation. Mean values are presented with their standard deviation between brackets. The effect of culture medium (CM) is indicated per fungal isolate by letter code.

Culture medium	PR growth		PP growth	
	mean (st.d.)	*CM*	mean (st.d.)	*CM*
100% CSI[1]	0.731 (0.047)	*abc*	0.819 (0.052)	*ab*
90% CSI + 10% sterile BHI[2]	0.689 (0.055)	*abcd*	0.738 (0.049)	*abc*
+ 10% Bv supernatant	0.553 (0.037)	*cd*	0.525 (0.021)	*e*
+ 10% Bv cell suspension	0.516 (0.069)	*d*	0.534 (0.017)	*de*
75% CSI + 25% sterile BHI	0.878(0.151)	*a*	0.726 (0.110)	*abcd*
+ 25% Bv supernatant	0.600 (0.093)	*bcd*	0.488 (0.045)	*e*
+ 25% Bv cell suspension	0.551 (0.074)	*cd*	0.466 (0.100)	*cde*

50% CSI + 50% sterile BHI	0.770 (0.067)	*ab*	0.845 (0.070)	*a*
+ 50% Bv supernatant	0.592 (0.066)	*bcd*	0.500 (0.056)	*e*

[1] CSI: corn silage infusion, [2] BHI: brain-heart infusion broth.

Over the nine culture media, PR and PP exhibited a different growth pattern. PR showed the highest OD_{620} on 75 % CSI with 25 % sterile BHI. The difference was not significant with 100 % CSI and with the 90 % and 50 % CSI complemented with sterile BHI, but was significant with all the media containing Bv supernatant or cell suspension. The OD_{620} of PP was the highest on 50 % CSI with 50 % sterile BHI, but just like for PR the differences were only significant with media containing Bv supernatant or cell suspension.

Per culture medium, only few differences in growth were detected between PR and PP. On 100 % CSI, PP grew significantly stronger than PR during the five days incubation period, while on 75 % CSI with 25 % sterile BHI the opposite was observed.

In the microtiter plate assay, Bv growth in CSI supplemented with 10% or 25% Bv cell suspension has also been monitored: the mean OD values were respectively 0.002 and 0.014 for 10 and 25% Bv cell suspension, so growth of Bv cells was poor.

2.1.2. Conidiospore survival and spore germination

Conidiospore survival in the different culture media was evaluated by streak-plating after 24 hours of incubation. Photographs were made of the obtained plates, shown in Figure 1.

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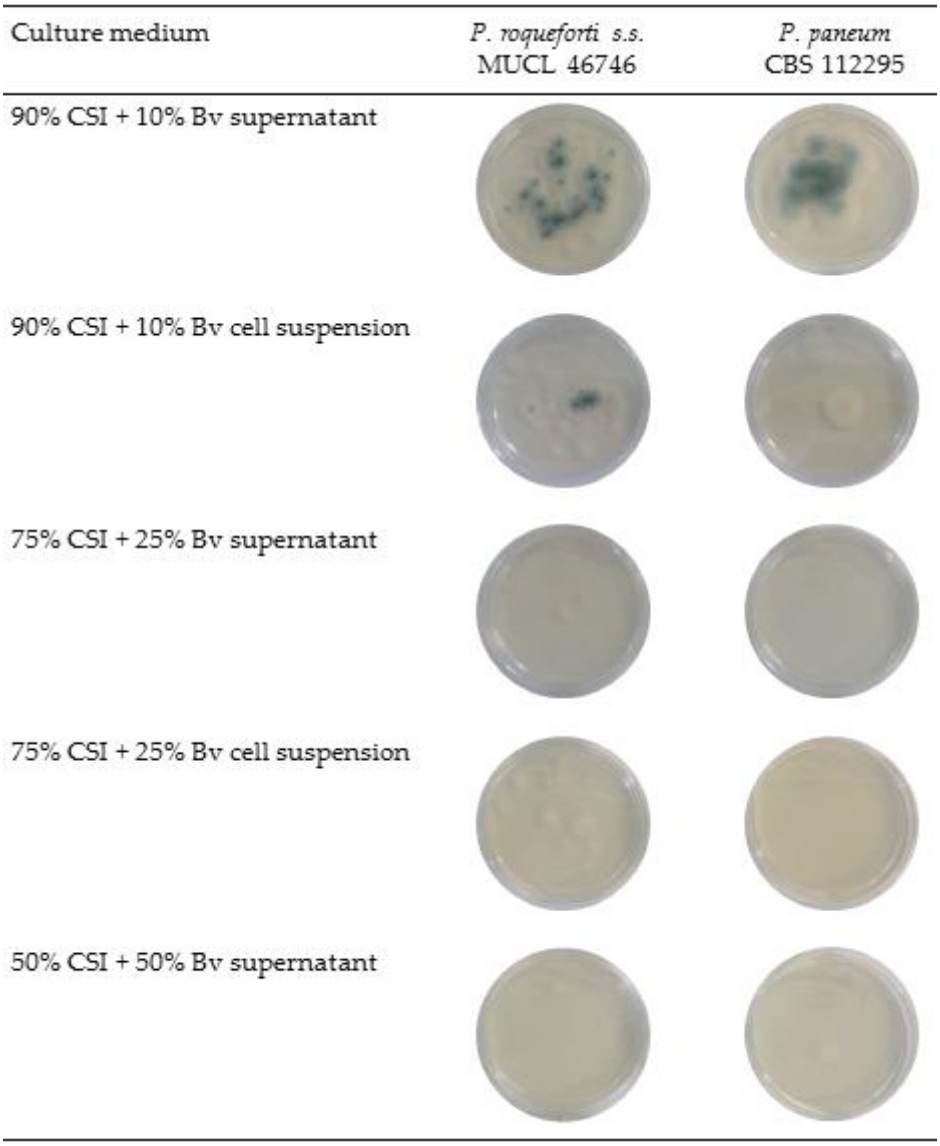

Figure 1. *In vitro* experiment evaluating the antagonistic effect of *B. velezensis* NRRL B-23189 (Bv) towards *P. roqueforti s.s. MUCL 46746* and *P. paneum* CBS 112295: streak-plates to monitor conidiospore survival after 24 hours of incubation.

Conidiospore survival varied considerably among the culture media: 90 % CSI in combination with 10 % of Bv supernatant facilitated the highest conidiospore survival, followed by 90 % CSI and 10 % Bv cell suspension. Inclusion of 25 % or 50 % of Bv supernatant or cell suspension in the medium resulted in lower conidiospore survival. A striking observation is that the PP conidiospores exhibited stronger growth on the streak-plates compared to PR conidiospores, confirming a lower growth rate of *P. roqueforti* s.s. [8,40].

The results of the conidiospore germination evaluation are presented in Table 2.

Table 2. *In vitro* experiment evaluating the antagonistic effect of *B. velezensis* NRRL B-23189 (Bv) towards *P. roqueforti s.s. MUCL 46746* (PR) and *P. paneum* CBS 112295 (PP): conidiospore germination (%) after 24 hours of incubation. Mean values are presented with their standard deviation between brackets. The effect of culture medium (CM) is indicated per fungal isolate by letter code.

Culture medium	PR conidiospore germination		PP conidiospore germination	
	mean (st.d.)	*CM*	mean (st.d.)	*CM*
100% CSI[1]	40 (2)	*a*	37 (3)	*a*
90% CSI + 10% sterile BHI[2]	23 (5)	*bc*	28 (4)	*ab*

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+ 10% Bv supernatant	34 (9)	*ab*	35 (7)	*a*
+ 10% Bv cell suspension	12 (2)	*d*	12 (9)	*de*
75% CSI + 25% sterile BHI	33 (3)	*ab*	23 (4)	*bc*
+ 25% Bv supernatant	17 (5)	*cd*	16 (4)	*cd*
+ 25% Bv cell suspension	15 (5)	*cd*	6 (3)	*e*
50% CSI + 50% sterile BHI	18 (4)	*cd*	19 (3)	*bcd*
+ 50% Bv supernatant	14 (2)	*cd*	13 (3)	*de*

[1] CSI: corn silage infusion, [2] BHI: brain-heart infusion.

For both PR and PP, the highest percentage of conidiospore germination after 24 hours was observed in 100 % CSI, illustrating their good adaptation to silage conditions. In CSI with Bv cell suspension added, in general low germination percentages were observed. Surprisingly, an increased conidiospore germination percentage for PR as well as PP was observed in 90 % CSI with 10 % Bv supernatant, but the difference with 90 % CSI with 10 % sterile BHI was not significant.

PR and PP conidiospore germination only differed significantly after 24 hours of incubation in 75 % CSI complemented with 25 % sterile BHI or 25 % Bv cell suspension: in both media, conidiospore germination of PP was significantly lower compared to PR.

2.1.3. Screening of roquefortine C production

The antagonistic effect of Bv against *P. roqueforti s.l.* has been confirmed on multiple levels (i.e. conidiospore germination and survival, and fungal growth), rendering the tested Bv strain an interesting candidate silage inoculant for *in vivo* inhibition of *P. roqueforti s.l.* growth in silages. However, it must be checked that growth inhibition of *P. roqueforti s.l.* by Bv does not trigger an increased mycotoxin production. Therefore, a quantitative screening of the production of the indicator mycotoxin roquefortine C (ROC) [1] during the five-days incubation period has been performed. These results are presented in Table 3.

Table 3. *In vitro* experiment evaluating the antagonistic activity of *Bacillus velezensis* NRRL B-23189 (Bv) towards *P. roqueforti s.s.* MUCL 46746 (PR) and *P. paneum* CBS 112295 (PP): screening of roquefortine C production (μg g^{-1} freeze-dried fungal biomass) after five days of incubation.

Culture medium	PR	PP
100% CSI[1]	4.68	1.18
90% CSI + 10% sterile BHI[2]	5.91	1.29
+ 10% Bv supernatant	6.80	1.22
+ 10% Bv cell suspension	5.46	1.71
75% CSI + 25% sterile BHI	10.92	2.72
+ 25% Bv supernatant	3.48	1.03
+ 25% Bv cell suspension	3.93	2.44
50% CSI + 50% sterile BHI	4.13	1.32
+ 50% Bv supernatant	2.51	1.48

[1] CSI: corn silage infusion, [2] BHI: brain-heart infusion

Addition of Bv supernatant or cell suspension generally did not result in elevated ROC production by any of the two *P. roqueforti s.l.* isolates compared to CSI in combination with sterile BHI. PP clearly produced less ROC than PR during the five-days incubation period. For PP, the highest ROC levels were detected in 75 % CSI with 25 % sterile BHI or Bv cell suspension, but ROC levels were not highly variable across the nine culture media.

For PR, addition of 10 % sterile BHI, Bv supernatant or cell suspension to 90 % CSI increased ROC production compared to 100 % CSI. Addition of 25 % sterile BHI intensified ROC production even further, while addition of 50 % BHI resulted in lower ROC levels compared to 25 % BHI. The highest ROC production by PR was detected on 75 % CSI with 25 % sterile BHI, which was also the culture medium exhibiting the strongest growth. Sterile BHI is a very nutritious culture medium. Due

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to Bv growth, the nutrient levels in the BHI in which the bacterium was cultured prior to the start of the experiment have dropped. Therefore, CSI supplemented with Bv supernatant or cell suspension contained less nutrients available for *P. roqueforti s.l.* growth and mycotoxin production. Since a positive correlation between growth of *P. roqueforti s.s.* and ROC production has been detected by Boichenko, *et al.* [41], this might explain the elevated ROC production by PR observed in the media containing 25 and 50 % sterile BHI: on these media the highest growth was registered in the present study.

2.2. In vivo experiment

The microtiter plate assay of the *in vitro* experiment showed poor growth of Bv cell suspension in CSI. To check if Bv cell suspension as a silage inoculant would be able to grow and produce cyclic lipopeptides reducing *P. roqueforti s.l.* numbers *in vivo* in grass – white clover silage artificially contaminated with PR or PP, a microsilo experiment was performed. Mean silo density was 188 kg dry matter (DM) per kg fresh matter (FM). After an ensiled period of 56 days, samples were taken for enumeration of *P. roqueforti s.l.* and for determination of DM content and pH. These results are presented in Table 4.

Table 4. Microsilo experiment with perennial ryegrass – white clover, artificially contaminated with *P. roqueforti s.s.* MUCL 46746 (PR) and *P. paneum* CBS 112295 (PP), to evaluate the antagonistic effect of *B. velezensis* NRRL B-23189 (Bv) cell suspension: *Penicillium roqueforti s.l.* counts, dry matter content and pH at desiling after 56 days. Mean values are presented with their standard deviation between brackets. Statistically significant effects of fungal contamination (FC) and additive (Add.) are indicated by letter codes. Significant interaction between these two factors is indicated by *-symbols, and the effect of one factor is determined per level of the other factor (designated by lettercodes without and with ‘-symbols).

OBJECTS		*P. roqueforti s.l.* (log_{10} spores g^{-1} FM)		Dry matter at desiling (g kg^{-1} FM)			pH at desiling		
FC.	Add.	mean (st.d.)	*FC.*	mean (st.d.)	*FC.**	*Add.**	mean (st.d.)	*FC.**	*Add.**
No	No	< 1.69	*a*	431 (2)	*a*	-	4.83 (0.05)	*a*	-
PR	No	2.08 (0.21)	*ab*	413 (0)	*b*	*a*	4.84 (0.04)	*a*	*a*
	Bv	1.79 (0.36)		377 (5)	*b′*	*b*	4.53 (0.06)	*a′*	*b*
PP	No	2.40 (0.19)	*b*	397 (3)	*c*	*a′*	4.62 (0.07)	*b*	*a′*
	Bv	2.32 (0.14)		402 (5)	*a′*	*a′*	4.53 (0.08)	*a′*	*a′*

P. roqueforti s.l. counts were not significantly influenced by additive application, but evidently artificial contamination had a significant influence: PP contaminated silage contained significantly more *P. roqueforti s.l.* propagules than non-contaminated silage.

For both DM and pH at desiling, a significant interaction between the factors contamination and additive was detected. The DM content of silage without additive was significantly lowered upon *P. roqueforti s.l.* contamination. Application of Bv cell suspension resulted in significantly lower DM content of PR contaminated silage compared to PP contaminated silage. Additive application had a significant effect on PR contaminated silage: no additive resulted in a significantly higher DM content compared to Bv cell suspension. Silage pH without additive application was significantly lowered by PP contamination compared to non-contaminated and PR contaminated silage. No significant effect of contamination on pH was detected upon application of Bv cell suspension. In PR contaminated silage, treatment with Bv cell suspension resulted in a significantly reduced pH compared to no additive. Since no literature data could be found reporting the effect of *B. velezensis* on silage fermentation characteristics, the explanation for the observed differences in DM content and pH between objects remains elusive.

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3. Discussion

It can be concluded from the *in vitro* experiment that both Bv supernatant and cell suspension had an inhibiting effect on *P. roqueforti s.l.* growth registered as OD_{620}. Chitarra, *et al.* [38] demonstrated that Bv culture supernatant had a negative effect on *P. roqueforti s.s.* conidiospore germination. The currently described *in vitro* experiment generally confirms this finding, but ascribes an even more potent inhibition of conidiospore germination to Bv cell suspension. The presence of either Bv supernatant or Bv cell suspension did not trigger an increased ROC production by *P. roqueforti s.l.*, which is crucial for a candidate silage additive.

Based on the results of the *in vitro* experiment, *B. velezensis* appears to be a promising antagonist towards *P. roqueforti s.s.* as well as *P. paneum*. Bv growth in CSI was however very limited despite aerobic incubation conditions. This can be due to the intrinsic nature of the species, but also to the fact that the strain was not adapted to acidic conditions prior to the experiment. To be able to successfully apply *B. velezensis* as a silage inoculant producing antifungal lipopeptides *in vivo* in silages, good bacterial growth in silage conditions is a prerequisite. *In vitro* in CSI, however, Bv growth was very poor. *B. velezensis* is an aerobic micro-organism [42,43]. Ruiz-Garcia, *et al.* [43] have found that *B. velezensis* can grow in the pH range of 5-10. For the *in vitro* experiment, CSI at pH 3.79 was used. This pH is well below pH 5, so the lack of Bv growth observed is very likely due to a too acidic growth medium. Velmurugan, *et al.* [37] have found that antifungal activity of *B. velezensis* remained stable in a pH range of 2-10 at 25 °C for 24 hours, but this was tested on culture supernatant and not on living bacteria. Taking the clear effect of Bv on conidiospore survival and germination *in vitro* into account, lipopeptides can be assumed to be present in the culture media – most likely introduced by the Bv supernatant or cell suspension at the start of the experiment. Monitoring of lipopeptide production should definitely be included in future experiments. Adding lipopeptides as a silage additive instead of a *Bacillus*-based inoculant would also be an option, prone to future research.

The Bv cell suspension applied to the *P. roqueforti s.l.* contaminated grass – white clover silage was not capable of living up to the great expectations which had arisen in the *in vitro* experiment: no significant reduction of *P. roqueforti s.l.* numbers compared to no additive application could be detected in silage. Most likely, this is due to a very short aerobic phase of the ensiling process in microsilos and a quick pH-drop, which are both beneficial for silage quality. Another option is that the manufacturing process of the Bv cell suspension was not optimal since it was no commercially available product resulting from extensive R&D. Moreover, since *Bacillus* species can carry antibiotic resistance genes [44,45], it is of the outmost importance to select strains not bearing these genes as potential silage inoculant candidates. The *B. velezensis* strain NRRL B-23189 has not been checked for the absence of antibiotic resistance genes.

In conclusion, the applied Bv cell suspension was unsuccessful in displaying antifungal properties towards *P. roqueforti s.l.* in the context of the *in vivo* microsilo experiment. However, it must be pointed out that the microsilos used for the *in vivo* experiment mimic silage fermentation conditions well, but the feed-out phase is not simulated [8]. During feed-out, aerobic metabolism re-flourishes, allowing yeasts and fungi (e.g. *P. roqueforti s.l.*) but also *Bacilli* to proliferate. *B. velezensis* enumeration as well as quantification of lipopeptides at different time points during the different phases of the ensiling process would definitely provide more information about the antagonistic potential of *B. velezensis* in a silage matrix.

It would be promising if a *B. velezensis* based silage inoculant could survive the ensiling process, producing antifungal lipopeptides *in vivo* during feed-out of silage. This would be an elegant strategy to prevent mycotoxin production by toxigenic fungi since multiple mycotoxin remediation strategies (e.g. mycotoxin binders, microbial degradation) are available when prevention has failed, but their *in vivo* efficacy is highly questionable [46-49]. Prevention efforts are definitely preferable [50].

4. Materials and Methods

4.1. P. roqueforti s.l. conidiospore suspensions

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Two fungal isolates were used for the experiments: *P. roqueforti s.s.* MUCL 46746 (PR) and *P. paneum* CBS 112295 (PP). For the conidiospore suspension preparation, monoconidial fungal inoculum is seeded in the center of 90-mm diameter Petri dishes with Potato Dextrose Agar (PDA, Sigma) in a laminar flow cabinet. The plates are aerobically incubated upright in the dark at 25 °C during 14 days. Conidia were harvested by washing with physiological water (0.85% sodium chloride, Sigma) containing 0.01% Tween 80 (Duchefa), with the aid of a sterile pipet tip. The suspension was transferred to a sterile centrifugation tube. After centrifugation at 8 500 rpm during 15 min, the supernatant was discarded and the conidiospore pellet was resuspended in sterile physiological water without Tween 80 added. The centrifugation and resuspension steps were repeated, after which the conidiospore suspension was filtered through a double layer of sterile miracloth (Millipore) to remove mycelial fragments and conidial aggregates. Conidiospore concentration was determined with a Bürker chamber and adjusted to the desirable concentration with sterile physiological water. When conidiospore suspensions were not readily used, 50% glycerol (Scharlau) was added and the suspensions were stored long-term at -80 °C.

4.2. In vitro experiment

4.2.1. Corn silage infusion

Whole crop maize silage (350 gram DM kg^{-1} FM, no roquefortine C detectable, ensiled for 50 days) was dried at 60 °C and milled to 1-mm particles. Corn silage infusion (CSI) was prepared as described by Niderkorn [51]: milled maize silage was infused in distilled water at 6 % (w/v) during two hours at 60 °C, followed by filtration through miracloth (Millipore) and a folded paper filter (Whatman 597 ½). After centrifugation at 10 000 rpm during 15 min, the pellet was discarded and the supernatant was collected. The pH of the supernatant was brought to the original pH of the silage (*i.e.* 3.79) using the same ratio of lactic acid (min. 99%, Fluka) to acetic acid (min. 99%, Sigma) as found in the silage (*i.e.* 3.17). The supernatant was subsequently sterilized through a syringe filter (cellulose acetate - 0.45 µm pore size - 25 mm diameter, GVS) and stored at 4 °C until use within 24 hours.

4.2.2. *B. velezensis* treatment solutions

B. velezensis NRRL B-23189 (Bv) was grown aerobically in the dark at 30 °C on Plate Count Agar (PCA, Sigma). Four-day old cultures were subcultured *in duplo* in 15 ml of Brain-Heart Infusion broth (BHI, Sigma) and aerobically stir-cultured in the dark at 30°C on a magnetic shaker at 130 rpm. Additionally, an additional 15-ml portion of sterile BHI was not inoculated with Bv. After 48 hours, one BHI-replicate inoculated with Bv was centrifuged during 15 min at 9 500 rpm. The Bv supernatant was collected and sterilized through a syringe filter (cellulose acetate - 0.45 µm pore size - 25 mm diameter, GVS). The other inoculated BHI-replicate was used as such, as Bv cell suspension (containing $8*10^7$ cfu ml^{-1}), as determined by streak-plating of a dilution series in physiological water on PCA. Sterile BHI was used as a negative control.

4.2.3. Experimental setup

By combining different volumes of sterile BHI, Bv supernatant or Bv cell suspension with CSI, nine different liquid culture media were prepared (vol/vol): 1) 100 % CSI, 2) 90 % CSI and 10 % sterile BHI, 3) 90 % CSI and 10 % Bv supernatant, 4) 90 % CSI and 10 % Bv cell suspension, 5) 75 % CSI and 25 % sterile BHI, 6) 75 % CSI and 25 % Bv supernatant, 7) 75 % CSI and 25 % Bv cell suspension, 8) 50 % CSI and 50 % sterile BHI, and 9) 50 % CSI and 50 % Bv supernatant.

The nine culture media were distributed *in duplo* into two sets of eighteen 15-ml falcon tubes, for infection with either PR or PP to a final conidiospore concentration of $1*10^4$ conidiospores ml^{-1} medium. The first set of falcon tubes was filled with 3 ml of medium and was used for a microtiter plate assay, as well as for monitoring of conidiospore germination and conidiospore survival. The second set of falcons was used for screening of ROC production. The empty weight of these falcons was noted to allow calculation of the freeze-dried mycelium weight, and all falcons were filled with 1 ml of medium.

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For the microtiter plate assay, 200 µl of culture medium was introduced per well (N=4 per object, except for 90 % CSI + 10 % sterile BHI and 75 % CSI + 25 % sterile BHI: N = 8). Two negative controls without *P. roqueforti s.l.* conidiospores (*i.e.* 90 % CSI + 10 % Bv cell suspension and 75 % CSI + 25 % Bv cell suspension) were included to enable monitoring of solely Bv growth in CSI, providing insight into its possible use as a silage inoculant. After sealing of the microtiter plate with respiratory foil, the plate was statically incubated in aerobic conditions in the dark for five days at 20 °C. The optical density was determined spectrophotometrically at 620 nm immediately after five days, with subtraction of the initial OD_{620} value per well immediately after filling.

Conidiospore survival in the different culture media was monitored after 24 hours by streak-plating 100-µl samples from the first set of falcon tubes (N=3) on PDA supplemented with 0.5% acetic acid (min. 99%, Sigma). All plates were aerobically incubated bottom-up in the dark at 20 °C and fungal development was evaluated after four days.

Conidiospore germination was evaluated after 24 hours of incubation on 20-µl samples (N=4) taken from the first set of falcon tubes. Per replicate, a 20-µl sample was placed on a glass slide cleaned with 70 % ethanol (Sigma), followed by covering with a clean cover slide and flame fixation. Randomly, 100 conidiospores were counted (evenly spread over the glass slide) and the percentage of germinated conidiospores was determined, using a phase-contrast microscope at 400x magnification (Motic). Conidiospores were considered to have germinated when the length of the germ tube exceeded one-half of the spore diameter.

The second set of falcon tubes was statically incubated in aerobic conditions during five days in the dark at 20 °C without shaking, and stored at -20 °C prior to freeze-drying with an Alpha 1-2 LD Plus lyophilizer (Christ) according to the manufacturer's guidance. After registration of the freeze-dried weight, ROC was quantified by LC-MS/MS (N=1).

4.2.4. Quantitative screening of roquefortine C production

Roquefortine C (ROC) was extracted with ethyl acetate and dichloromethane (both min. 99.5%, Acros Organics) as described by Delmulle [52], and quantified based on the method described by Monbaliu, *et al.* [53]. LC-MS/MS analysis was performed with a Waters Acquity UPLC system coupled to a Micromass Quattro Premier XE triple-quadrupole mass spectrometer (Waters), equipped with Masslynx software for data processing. A 150 mm x 2.1 mm reverse-phase C18 column was used, with a 10 mm x 2.1 mm guard column of the same material (resp. 5 and 3.5 µm inner diameter, Waters). The column was kept at room temperature. The mobile phase consisted of variable mixtures of mobile phase A (water/methanol/acetic acid, 94/5/1 (v/v/v) and 5 mM ammonium acetate) and mobile phase B (methanol/water/acetic acid, 97/2/1 (v/v/v) and 5 mM ammonium acetate) at a flow rate of 0.3 ml/min with a gradient elution program, mentioned in Table 5.

Table 5. Gradient elution program for quantification of roquefortine C by LC-MS/MS.

Time (min)	% mobile phase A	% mobile phase B
0-6	95	5
6-10	35	65
10-11	1	99
11-14	95	5
14-15	25	75
15-16	1	99
16-18	95	5
18-19	25	75
19-20	1	99
20-29	95	5

The injection solvent consisted of mobile phase A/mobile phase B (60/40, v/v) and 5 mM ammonium acetate. The injection volume of the samples on the analytical column was 20 µl. The mass spectrometer was operated in the positive electrospray ionization (ESI+ mode). Capillary

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voltage was 3.2 kV. High-purity nitrogen was used as drying and ionization (ESI+) nebulizing gas, and argon was used as collision gas for collision-induced dissociation. Source and desolvation temperatures were set at 150 and 350 °C respectively. ROC was analyzed using selected reaction monitoring (SRM). The method was validated according to Commission Decision 2002/657/EC.ROC is expressed relatively to a known amount of internal standard, added to all samples: 0.2 ng of zearalanone (ZAN). To allow quantification of ROC, four reference samples (i.e. ethyl acetate) were spiked with ROC (negative control and 3 known amounts). Based on the response factors (i.e. peak area for ROC / peak area for ZAN) for these reference samples, a linear regression was determined per run and used for the quantification of ROC in the experimental samples. ROC eluted after approx. 7.8 min, while ZAN had a retention time of approx.9.2 min. In Masslynx, the peak areas for both mycotoxins was determined and the response factor was calculated for each sample. Based on the four spiked reference samples per run, a linear regression was fitted for quantification of the samples with unknown ROC content. The decision limit was 5 ng per ml, while quantification was possible from 10 ng per ml injection solution. Quantification of ROC by LC-MS/MS comprised quantification of predominantly ROC, in combination with its stereo-isomer formed under acidic, basic or photochemical conditions, and with roquefortine D [54].

4.3. In vivo experiment

For the *in vivo* experiment, microsilos with a content of 2.75 liter were use, equipped with a CO_2 slot preventing air ingress but allowing fermentation gasses to escape. Every microsilo has a unique number for identification [8].

A mixture of perennial ryegrass and white clover (second cut) was mown and prewilted in the field to 420 gram dry matter (DM) per kg fresh matter (FM). After chopping with a New Holland precision chopper to 10-12 cm particles, the starting material was homogenized well prior to ensiling of the different objects: 1) no infection, no additive, 2) PR, no additive, 3) PP, no additive, 4) PR, Bv cell suspension, and 5) PP, Bv cell suspension.

P. roqueforti s.l. conidiospore suspensions were freshly prepared as described in section 4.1.

Bv cell suspension was obtained after a three-days incubation period of *B. velezensis* NRRL B-23189 in 100 ml of BHI at 30 °C on a rotary shaker at 130 rpm. After centrifugation at 10 000 rpm during 5 min, the supernatant was discarded and the bacterial pellet was resuspended in 50 ml physiological water containing 16 % glycerol (Scharlau) and stored at -80 °C. Just before ensiling, the cell suspension was defrosted at 20 °C. The concentration of the obtained Bv cell suspension was determined by streak-plating 100 µl aliquots of a decimal dilution series on PCA (N=3). PCA plates were incubated aerobically at 30 °C for four days and *B. velezensis* was enumerated, taking the appropriate dilution factor into account: $5*10^5$ cfu of *B. velezensis* were present per milliliter.

Per object, the fresh feed commodity was spread evenly in a thin layer onto a polyethylene sheet and sprayed with an equal amount of treatment solution using handheld sprayers. Object 1 was sprayed with 20 ml of sterile physiological water per kg FM. Per kg FM, 10 ml of PR or PP conidiospore suspension was applied to objects 2 to 5 (infection at 500 conidiospores per gram FM), as well as 10 ml of the appropriate additive solution: either sterile physiological water (objects 2 and 3), either Bv cell suspension (objects 4 and 5, infection at 5 000 cfu per gram FM). The microsilos were filled in two stages using a hydro-pneumatic press. The empty weight of each microsilo was noted before filling and after filling to determine the mean silo density. After 56 days, the microsilos were desiled. The upper and lower 3-5 cm of silage was removed, and the remaining silage was homogenized prior to sampling for determination of pH [55] and dry matter content (by air draying at 65 °C) and for *P. roqueforti s.l.* enumeration.

To enumerate the amount of *P. roqueforti s.l.* propagules in a sample, exactly 20 grams of fresh matter was brought in a stomacher bag with lateral filter (Interscience) along with 90 ml of sterile physiological water supplemented with 0.01 % Tween 80. After placing the bag in a stomacher (Seward) for homogenization during 1 min at 200 rpm, a decimal dilution series was prepared in physiological water. From this dilution series, 100 µl was streak-plated on Petri dishes containing PDA supplemented with 0.5% acetic acid [56], using a sterile Drigalsky spatula (N=3 per dilution).

The plates were aerobically incubated bottom-up in the dark at 20 °C. After five days of incubation, the number of *P. roqueforti s.l.* propagules was counted at the appropriate dilution (i.e. propagule number below 50 per plate). The mean value of the three readings was determined and the number of fungal propagules in the original sample was calculated taking the particular dilution factor into account. *P. roqueforti s.l.* counts were transformed to a logarithmic scale.

4.4. Statistical analysis

The obtained data were statistically analyzed with the SPSS Statistics 24 program. Significance was declared at 95%, with $p < 0.05$. Per parameter, normality was checked by Shapiro-Wilk's test (applying Bonferroni correction) and homoscedasticity was checked with Levene's test. A multiple Anova was performed to check for significant interaction between factors. In case of significant interaction, an Anova analysis was performed for each level of one factor to assess the effect of the other factor's). If variances were equal for normally distributed variables, Anova with Tukey as *post hoc* test was performed, otherwise a Welch Anova with Dunnett T3 as *post hoc* test was executed. Not normally distributed parameters were subjected to a non-parametric test according to Kruskal-Wallis with Dunn's test for pairwise comparisons (applying Bonferroni correction). In case of no significant interactions between factors and homoscedasticity, the main effects of the factors were determined likewise over all levels of the other factor(s).

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